

## Weak Effect of Membrane Diffusion on the Rate of Receptor Accumulation at Adhesive Contacts

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**ABSTRACT** To assess if membrane diffusion could affect the kinetics of receptor recruitment at adhesive contacts, we transfected neurons with green fluorescent protein-tagged immunoglobulin cell adhesion molecules of varying length (25–180 kD), and measured the lateral mobility of single quantum dots bound to those receptors at the cell surface. The diffusion coefficient varied within a physiological range (0.1–0.5  $\mu\text{m}^2/\text{s}$ ), and was inversely proportional to the size of the receptor. We then triggered adhesive contact formation by placing anti-green fluorescent protein-coated microspheres on growth cones using optical tweezers, and measured surface receptor recruitment around microspheres by time-lapse fluorescence imaging. The accumulation rate was rather insensitive to the type of receptor, suggesting that the long-range membrane diffusion of immunoglobulin cell adhesion molecules is not a limiting step in the initiation of neuronal contacts.

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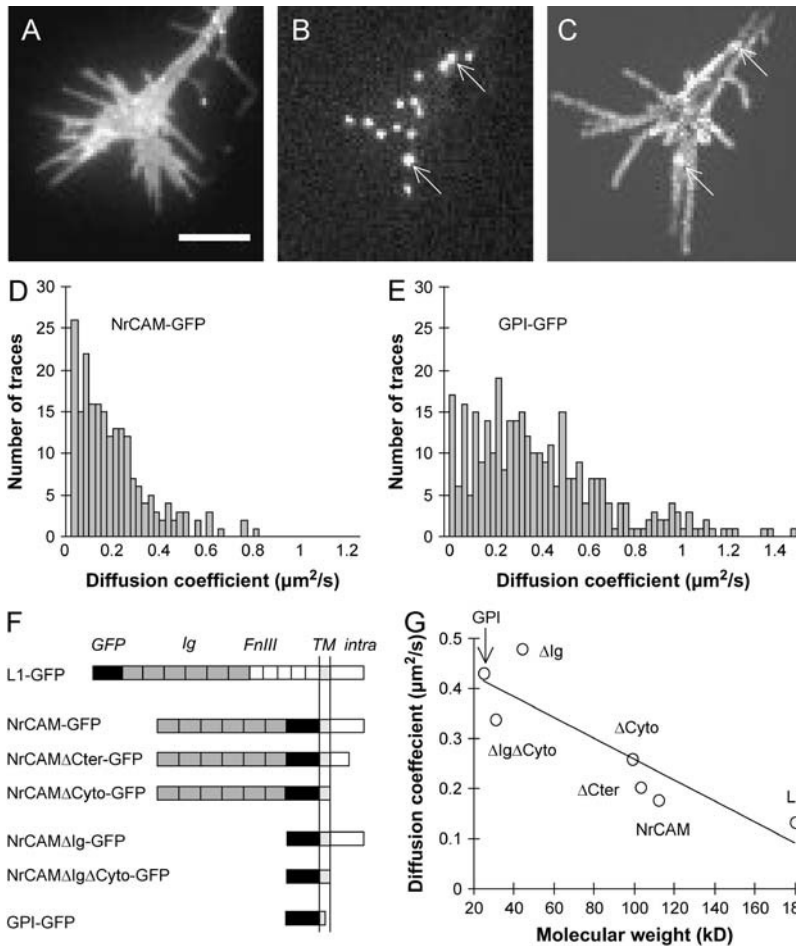
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The formation of adhesive contacts between cells is fundamental in biology. It involves specific adhesion proteins, e.g., IgCAMs, which are implicated in neurite elongation and growth cone guidance (1). Contacts are initiated when adhesion molecules find counterreceptors on the surface of neighboring cells and make selective protein-protein bonds. Such interactions depend on the abundance of receptors expressed by the cells, but also on the ability of receptors to diffuse in the cell membrane (2). The regulation of receptor mobility by cytoplasmic partners, e.g., between L1/neurofascin and ankyrin (3,4), may then tune the rate at which adhesions form.

To assess if diffusion could affect the kinetics of receptor recruitment at adhesive sites, we used constructs of varying length (25–180 kD), all tagged extracellularly with green fluorescent protein (GFP). These include L1-GFP, several truncated forms of neuronal-related cell adhesion molecule (NrCAM)-GFP (5), and glycosylphosphatidylinositol (GPI)-GFP (Fig. 1 *F*). We reasoned that size differences should result in contrasting lateral mobilities. To measure the diffusion coefficient of these receptors, we transfected primary culture neurons and labeled individual receptors with quantum dots (QD). Active growth cones were selected for the recordings (Fig. 1 *A*), since these structures are implicated in IgCAM-based locomotion and cell recognition; ~40% of the receptors were expressed at the plasma membrane (Table 1), allowing QD to bind specifically to transfected cells (Fig. 1 *B*). QD attached to the cell surface and moved in two dimensions, exploring the entire growth cone surface (Fig. 1 *C*). QD showed a variety of behaviors, some moving fast, others staying almost immobile. We tracked individual QD and calculated an instantaneous diffusion coefficient for each trajectory.

We thereby obtained a distribution of diffusion coefficients for each construct (Fig. 1, *D* and *E*) in the range of 0.1–1  $\mu\text{m}^2/\text{s}$  (6). As receptor size diminishes, the distribution shifts to higher mobility values, resulting in a clear inverse relationship between the molecular weight of the receptor and its average diffusion coefficient (Fig. 1 *G*). Since these receptors interact similarly with lipid microdomains (5), differences in mobility are unlikely to be associated with variations in the lipid environment. Truncations of intracellular regions caused a slight decrease in lateral mobility (7), which may be attributed to trapping of L1 or NrCAM cytoplasmic tail within the membrane scaffold, or to specific interactions with cytoskeletal partners such as ankyrin or SAP102 (3,4). Deletions of extracellular regions (fibronectin type III, immunoglobulin (Ig), or both) strongly reduced receptor diffusion (8). This may be due to steric effects linked to the high glycosylation levels of L1 and NrCAM ectodomains. Alternatively, IgCAMs with intact fibronectin type III and/or Ig domains are able to interact *cis* with themselves or other receptors (1), thus forming complexes with lower diffusion properties.

We then mimicked adhesive contacts using anti-GFP-coated latex microspheres, which selectively bound to transfected cells (Fig. 2, *A* and *B*; Table 1) and recruited GFP-tagged membrane receptors (Fig. 2, *C* and *D*). We placed microspheres on growth cones using optical tweezers, and followed the accumulation of receptors around them (Fig. 2 *E*). We quantified the ratio between the fluorescence level on the microsphere and that on adjacent regions. This enrichment factor increased in a few minutes, slightly faster



**FIGURE 1** Lateral mobility of GFP tagged receptors. Growth cone expressing NrCAM-GFP (A), labeled with anti-GFP conjugated QD (B). (C) Image of the maximum intensity from the QD channel detected for each pixel along a 1 min sequence, representing the global area explored by QD. Arrows indicate immobile QD. (D) Distributions of the diffusion coefficients for NrCAM-GFP and GPI-GFP. (E) Diagram of the various receptors. In all NrCAM constructs, the fibronectin type III domains have been replaced by GFP:  $\Delta$ Cter is deleted of the ankyrin binding motif, and downstream,  $\Delta$ Cyto of the entire cytoplasmic tail,  $\Delta$ Ig of the immunoglobulin domains, and  $\Delta$ Ig $\Delta$ Cyto of both extracellular and intracellular regions. (F) Average diffusion coefficient versus the molecular weight of each construct. The straight line is a linear fit ( $r = 0.88$ ). Bar = 5  $\mu$ m.

for smaller receptors (Fig. 2 F), and reached a plateau around 3 with minor differences between the constructs (Table 1). That equilibrium value corresponded to the saturation of antibody binding sites on microspheres by GFP-tagged receptors.

We modeled the receptor recruitment data using first-order kinetics:  $dC/dt = k_{on}(R - C)(L - C) - k_{off}C$ , where  $R$  is the receptor density at the cell surface ( $\approx 1000/\mu\text{m}^2$ ),  $L$  the density of GFP binding sites on microspheres ( $\approx 4000/\mu\text{m}^2$ ),  $C$  the surface density of bonds between antibodies and receptors, and  $k_{on}$  and  $k_{off}$  the forward and reverse rate

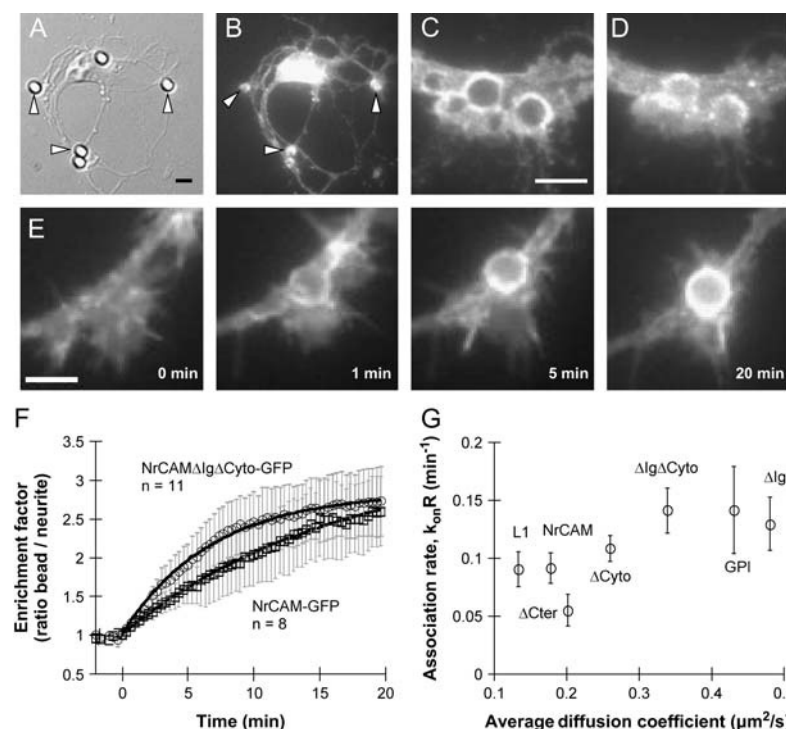
constants, respectively. Fluorescence measurements outside bead contacts indicated that there was no receptor depletion, so we took  $(R - C) = R$ . Furthermore, antibody-antigen bonds being very stable, we set  $k_{off} = 0$ . This left Eq. 1:  $C(t) = L[1 - \exp(-k_{on}Rt)]$ , which was used to fit the data and gave the two parameters  $R/L$  (Table 1) and  $k_{on}R$ .

The association rate  $k_{on}R$  increased weakly with the receptor diffusion coefficient (Fig. 2 C), showing that receptor accumulation at microsphere contacts is not diffusion-limited. This agreed with a theoretical model taking into

**TABLE 1** Surface expression, binding and recruitment of GFP-tagged receptors

Construct	L1	NrCAM	$\Delta$ Cter	$\Delta$ Cyto	$\Delta$ Ig	$\Delta$ Ig $\Delta$ Cyto	GPI	GFP
Surface fraction* (%)	35 $\pm$ 7 (14)	46 $\pm$ 10 (12)	34 $\pm$ 8 (16)	40 $\pm$ 4 (10)	46 $\pm$ 6 (16)	44 $\pm$ 12 (14)	48 $\pm$ 8 (15)	4 $\pm$ 3 (16)
No. beads per cell*	10.1 $\pm$ 1.0 (40)	4.5 $\pm$ 1.2 (25)	5.7 $\pm$ 0.9 (47)	4.5 $\pm$ 0.8 (34)	5.6 $\pm$ 1.0 (28)	7.1 $\pm$ 1.3 (79)	7.8 $\pm$ 1.8 (67)	0.7 $\pm$ 0.1 (38)
Enrichment factor <sup>†</sup>	2.6 $\pm$ 0.2 (46)	2.9 $\pm$ 0.2 (37)	3.0 $\pm$ 0.1 (67)	2.5 $\pm$ 0.2 (17)	2.8 $\pm$ 0.1 (47)	2.8 $\pm$ 0.1 (95)	2.9 $\pm$ 0.1 (108)	1.3 $\pm$ 0.1 (18)
Ratio $R/L$ (%)	22 $\pm$ 6 (9)	24 $\pm$ 7 (11)	18 $\pm$ 5 (7)	26 $\pm$ 6 (9)	26 $\pm$ 4 (8)	25 $\pm$ 8 (8)	25 $\pm$ 5 (10)	2 $\pm$ 1 (9)

All data are expressed as mean  $\pm$  SE, where ( $n$ ) is the number of cells\* or beads<sup>†</sup> examined in each condition. All GFP-tagged receptors are similarly expressed at the cell surface and bind to microspheres, in contrast with GFP alone, which remains intracellular.



**FIGURE 2** Kinetics of GFP-tagged receptor trapping. (A–D) Neurons transfected for NrCAM-GFP were incubated for 1 h with 4  $\mu\text{m}$  anti-GFP-coated microspheres. (A) Differential interference contrast image; (B) GFP channel. Arrowheads indicate bound beads that have recruited NrCAM-GFP. (C and D) Higher magnification views showing the recruitment of NrCAM-GFP (C), and a corresponding surface immunostaining, using an antibody to the hemagglutinin tag located at the N-terminus of NrCAM-GFP (D). (E) Time sequence of NrCAM-GFP accumulation around a microsphere placed on a growth cone for 10 s at time zero. (F) Individual data showing the enrichment factor versus time for NrCAM-GFP and NrCAM $\Delta\text{Ig}\Delta\text{Cyto}$ -GFP (mean  $\pm$  SE). The plain curves represent fits with Eq. 1. (G) Rate constant  $k_{\text{on}}R$  versus the diffusion coefficient for all receptors ( $n = 8$ –12 experiments for each construct). Bars = 5  $\mu\text{m}$ .

account the long-range diffusion of receptors toward a narrow zone where they can be irreversibly trapped by immobilized ligands (9). Beads coated with lower affinity ligands such as monoclonal antibodies against GFP (not shown), transient adhesion glycoprotein 1 (5), or N-cadherin (10) all induced slower accumulation of counterreceptors, suggesting that the adhesive reaction is the limiting step there. Thus, there appears to be a large enough reservoir of highly diffusive IgCAMs that can be mobilized quickly at adhesive sites, waiting for ligand binding. It is still possible that subtle differences in the diffusion of less mobile receptor complexes, controlled locally by the cytoskeleton or the lipid environment, can modulate the initiation and durability of neuronal interactions.

## EXPERIMENTAL METHODS

Methods provided as online supplemental material can be found by visiting BJ Online at <http://www.biophysj.org>.

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